

Glucocorticoids Modulate MicroRNA Expression and Processing during Lymphocyte Apoptosis^{*[S]}

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Glucocorticoids modulate immune development and function through the induction of lymphocyte apoptosis via mechanisms requiring alterations in gene expression. Recently, short, noncoding, microRNAs have been identified as key regulators of lymphocyte function; however, it is unknown whether glucocorticoids regulate noncoding microRNAs and whether this regulation contributes to lymphocyte apoptosis. We now show by both microarray and deep sequencing analysis that microRNAs are substantially repressed during glucocorticoid-induced apoptosis of primary rat thymocytes. Mechanistic studies revealed that primary microRNA transcripts were not repressed, whereas the expression of the key microRNA processing enzymes: Dicer, Drosha, and DGCR8/Pasha, were significantly reduced at both the mRNA and protein levels during glucocorticoid-induced apoptosis. To delineate the role of Dicer depletion and microRNA repression in apoptosis, we silenced Dicer expression in two human leukemic cell lines and demonstrated that Dicer depletion significantly enhanced glucocorticoid-induced apoptosis in both model systems. Finally, *in vitro* and *in vivo* overexpression of the conserved miR-17–92 polycistron, which was repressed significantly by dexamethasone treatment in both our microarray and deep sequencing studies, blunted glucocorticoid-induced apoptosis. These studies provide evidence of altered post-transcriptional microRNA expression and the repression of the microRNA bioprocessing pathway during glucocorticoid-induced apoptosis of lymphocytes, suggesting a role for microRNA processors and specific microRNAs in cell life/death decisions.

Recently, noncoding microRNAs have emerged as important gene expression regulatory elements. Transcribed by RNA polymerase II, microRNA precursors undergo extensive post-transcriptional processing by the nuclear RNase III enzyme Drosha and its co-factor DGCR8 (DiGeorge syndrome critical region 8)/Pasha followed by Ran-GTP/Exportin-5-dependent nuclear export. In the cytoplasm, primary microRNAs are further cleaved by the RNase III enzyme Dicer to yield a microRNA duplex. The biologically active, mature, single-

stranded microRNA is then incorporated into the microRNA-induced silencing complex. The microRNA-bearing microRNA-induced silencing complex proceeds to affect gene expression through the inhibitory engagement of complementary “seed sequences” within the 3′-UTRs of target mRNAs, resulting in translational inhibition (1, 2).

Through the modulation of gene expression, microRNAs are key effectors of fundamental biological processes including development, differentiation, and apoptosis (3). Apoptosis of lymphocytes is a highly coordinated process governing the development of the T-cell repertoire during thymocyte selection as well as the emergence of lymphoproliferative disease caused by the escape of malignant cells from apoptotic constraint. Recently, microRNAs have been implicated in both the regulation of lymphocyte apoptosis and the development of hematological malignancy. For example, hsa-miR-15 and -16 are strongly down-regulated in chronic lymphocytic leukemia and the expression of the anti-apoptotic oncogene Bcl-2 is inversely correlated with the expression of these microRNAs (4, 5). Furthermore, overexpression of hsa-miR-15 and -16 in a leukemic cell line resulted in the reduced expression of Bcl-2 and the induction of apoptosis, suggesting a pro-apoptotic tumor suppressor function for microRNAs 15 and 16 in human lymphocytes (6). Conversely, members of the hsa-miR-17–92 polycistron are up-regulated in B-cell lymphomas. Adoptive transfer of hematopoietic stem cells bearing a truncated portion of the mmu-miR-17–92 polycistron in c-Myc transgenic mice resulted in the rapid onset of malignant lymphomas. These lymphomas exhibited resistance to apoptosis and increased proliferation (7). Transgenic overexpression of the entire hsa-miR-17–92 polycistron in the murine hematopoietic compartment led to the development of lymphoproliferative disease and increased lethality. Lymphocytes derived from these animals displayed reduced sensitivity to Fas ligand-induced cell death as well as increased proliferation (8). Finally, the expression of the pro-apoptotic proteins, Bim² and phosphatase and tensin homolog, was decreased in response to hsa-miR-17–92 overexpression, suggesting a conserved, anti-apoptotic function for the miR-17–92 polycistron in lymphocytes.

Glucocorticoids are key effectors of lymphocyte development through the induction of apoptosis and the mutual antagonism of T-cell receptor activation-induced apoptosis during thymocyte selection (9). Because of their ability to induce rapid

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² The abbreviations used are: Bim, Bcl-2 interacting mediator of cell death; GR, glucocorticoid receptor; miRNA, microRNA.

apoptosis of lymphocytes, synthetic glucocorticoids are also critical components of hematolymphoid chemotherapy regimens. Glucocorticoid-induced apoptosis of lymphocytes is a multifaceted process, requiring signaling through the glucocorticoid receptor (GR) and the subsequent transactivation of apoptotic effector genes (10–13). The requirement for *de novo* transcriptional events in the execution of glucocorticoid-induced apoptosis is well established. However, it is not clear whether the expression of microRNAs is altered during glucocorticoid-induced apoptosis of lymphocytes and, if so, whether such alterations contribute to the cell death process. Here, we provide evidence of prevalent post-transcriptional repression of microRNA expression during glucocorticoid-induced apoptosis of primary rat thymocytes and further demonstrate the repression of the microRNA bioprocessors, Dicer, Drosha, and DGCR8/Pasha. Additionally, the stable depletion of Dicer in glucocorticoid-sensitive leukemic cell lines significantly enhanced glucocorticoid-induced apoptosis and overexpression of the glucocorticoid-repressed hsa-miR-17–92 polycistron-blunted glucocorticoid-induced apoptosis, indicating that the repression of microRNA expression and processing contributes to the progression of glucocorticoid-induced lymphocyte apoptosis.

EXPERIMENTAL PROCEDURES

Rat Primary Thymocyte Isolation—Rat primary thymocytes were isolated from adrenalectomized male Sprague-Dawley rats (60–75 g) ~1–2 weeks after surgery. Following decapitation, the thymi of three animals were removed and pooled in RPMI 1640 medium containing 10% heat-inactivated fetal bovine serum, 4 mM glutamine, 75 units/ml streptomycin, and 100 units/ml penicillin. Thymi were gently sheared with surgical scissors at room temperature. Sheared cells were filtered through 200-micron nylon mesh twice and centrifuged at $3,000 \times g$ for 5 min at room temperature. The cell pellet was then resuspended in fresh media and filtered into a sterile conical tube. The cells were cultured at a final concentration of 2×10^6 cells/ml and incubated at 37 °C, 5% CO₂ atmosphere.

Mouse Primary Thymocyte Isolation—Primary thymocytes were isolated from adrenalectomized male B57BL/6 wild-type control or hsa-miR-17–92 transgenic mice at 20 weeks of age. Following cervical dislocation, individual thymi were removed and gently sheared to dissociate free thymocytes. Sheared cells were filtered through 200-micron nylon mesh twice and centrifuged at $3,000 \times g$ for 5 min at room temperature. The cell pellet was then resuspended in fresh medium and filtered into a sterile conical tube. The cells were cultured at a final concentration of 2×10^6 cells/ml and incubated at 37 °C, 5% CO₂ atmosphere.

Human Cell Culture—The Jurkat GR α cell line was kindly generated by Dr. Nick Lu in our laboratory. Briefly, parental Jurkat cells were transfected with pTRE hGR α vector (14) using the Lonza Nucleofector System (Basel, Switzerland). Stably overexpressing clones were established by hygromycin/G418 selective pressure (200 μ g/ml). CEM-C7 leukemic cells were obtained from Dr. Carl Bortner. For all experiments, Jurkat GR α and CEM-C7 cells were cultured at a density of 2×10^5

cells/ml in RPMI 1640 medium containing 10% heat-inactivated fetal bovine serum, 4 mM glutamine, 75 units/ml streptomycin, and 100 units/ml penicillin.

Dicer Depleted Cells—CEM-C7 and Jurkat GR α cells were transduced with lentivirus encoding Dicer shRNA or nontargeting control at a multiplicity of infection of 5. Stable Dicer depleted cells were established by puromycin selection (2 μ g/ml). Anti-Dicer and nontargeting control Mission shRNAs were purchased from Sigma-Aldrich.

miR-17–92-overexpressing Cells—Jurkat GR α cells were transduced with lentivirus encoding hsa-miR-17–92 pre-miRNA or empty vector control at a multiplicity of infection of 10. Transduction at this multiplicity of infection resulted in a transduction efficiency of >90% as determined by CopGFP expression. Lenti-miR hsa-miR-17–92 precursor and empty vector clones were purchased from System Biosciences (Mountain View, CA).

Lentivirus Generation—All of the lentiviruses were packaged in HEK293T cells according to established protocols (15). HEK293T cells were transiently transfected with pMD2G, psPAX2, and transfer vector containing the desired gene or miRNA using Lipofectamine 2000. Supernatant was collected 48 h post-transfection and concentrated by centrifugation at $50,000 \times g$ for 2 h. The pellets were resuspended in PBS and used for infection. All of the titers were determined by quantitative PCR analysis of lentiviral integration into the host genome. In addition, titration of viruses co-expressing fluorescent moieties was determined by flow cytometric analysis.

MicroRNA Microarray—Rat primary thymocytes were isolated and cultured in the presence or absence of 100 nM dexamethasone for 6 h. Following treatment, total RNA was isolated from untreated control and dexamethasone-treated samples and subjected to microarray analysis using Agilent rat miRNA V2 (design identification 019159) 8×15 multiplex format arrays (Agilent Technologies) following the Agilent one-color miRNA complete labeling and hybridization kit protocol. Starting with 70–100 ng of total RNA, the cytidine bisphosphate reagent was used to selectively label mature miRNAs according to manufacturer's protocol. In combination with the miRNA microarray probe design, the cytidine bisphosphate reagent ensures detection of both low abundance and highly homologous miRNAs. Each labeled sample was hybridized for 20 h in a rotating hybridization oven. The slides were washed and then scanned with an Agilent scanner. The data were obtained using the Agilent feature extraction software (v9.5), using the miRNA defaults for all parameters. The Agilent feature extraction software performed error modeling, adjusting for additive and multiplicative noise. The resulting data were processed using the Rosetta Resolver[®] system (version 7.2) (Rosetta Biosoftware, Kirkland, WA). Error-weighted ratios and the associated *p* values were generated by Resolver to identify differentially expressed probes. A *p* value of <0.05 was used to generate gene lists of differentially expressed microRNAs.

MicroRNA Deep Sequencing—Rat primary thymocytes were isolated and cultured in the presence or absence of 100 nM dexamethasone for 6 h. Following treatment, total RNA was isolated from untreated control and dexamethasone-treated samples

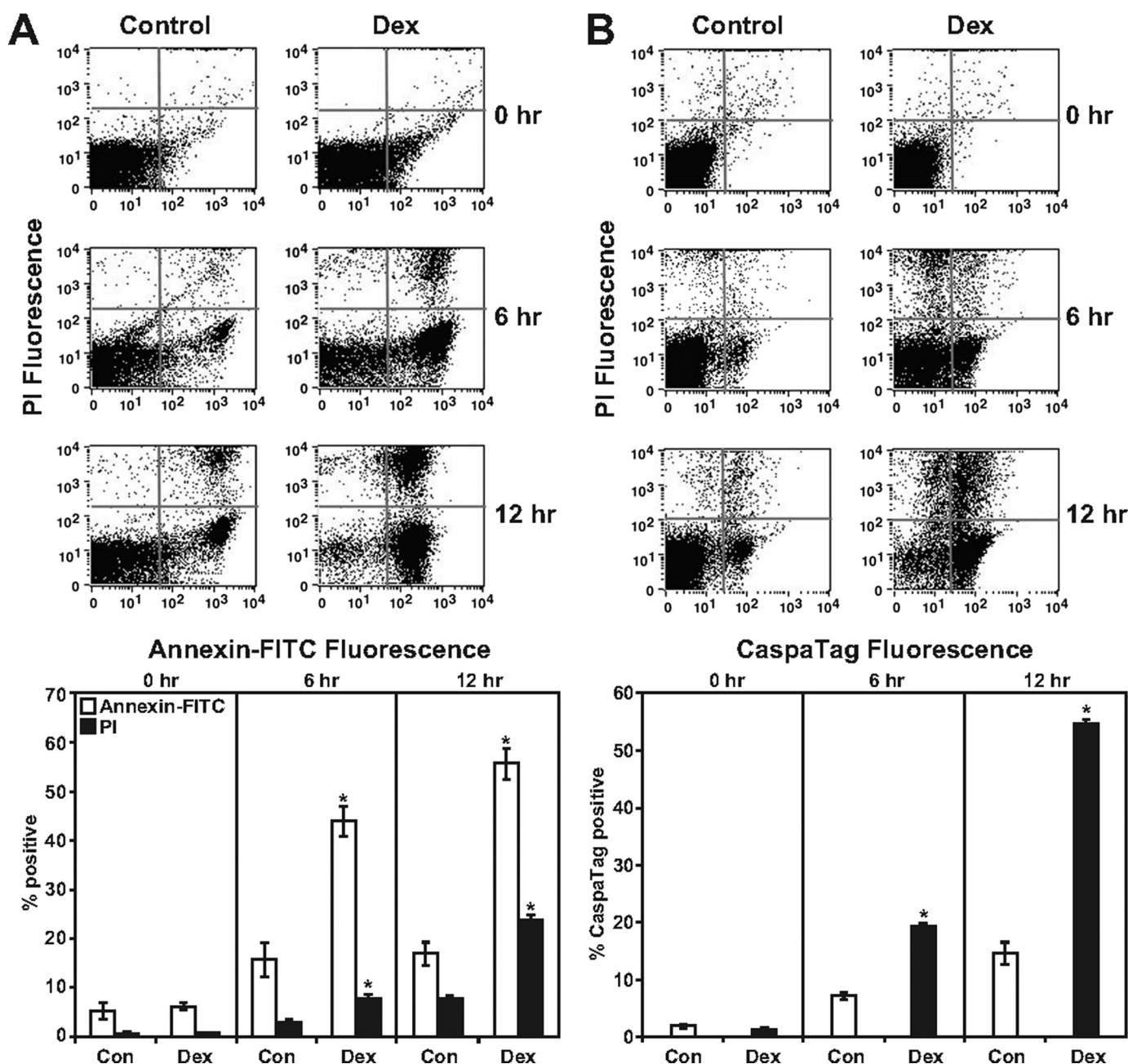


FIGURE 1. Evaluation of glucocorticoid-induced apoptosis of primary thymocytes. Primary thymocytes were isolated and treated with 100 nM dexamethasone (Dex) for 0, 6, or 12 h. Apoptotic progression was monitored via flow cytometric analysis of phosphatidylserine exposure, plasma membrane integrity, and caspase-3 activation. *A*, as cells undergo apoptosis, the internal phosphatidylserine component of the plasma membrane becomes externalized. This increased externalization was detected via fluorescently labeled annexin V. Additionally, the cells undergoing apoptosis exhibit a decrease in plasma membrane integrity. This loss of plasma membrane integrity was detected via the uptake of propidium iodide (PI) vital dye. The results are expressed as percentages of positive mean values \pm S.E. for three independent experiments. *, $p \geq 0.05$. *B*, apoptosis culminates in the activation of the effector caspase, caspase-3. Caspase-3 activation was monitored via the binding of fluorescently labeled CaspaTag to active caspase-3. The results are expressed as percentage positive mean values \pm S.E. for three independent experiments. *, $p \geq 0.05$. Con, control.

and subjected to microRNA deep sequencing. Small RNA cDNA libraries were prepared according to the manufacturer's protocol (small RNA sample prep kit, oligonucleotide only, protocol 71003; Illumina, Inc., San Diego, CA). Small RNA cDNA libraries were then sequenced according to the manufacturer's instructions on the Illumina Genome Analyzer II.

Bioinformatics—Reads were aligned to the reference rat genome (2004 assembly) using Bowtie (16). Given that mature

microRNAs are usually ~18–20 nucleotides in length, we used the first 20 nucleotides from the 5' end of the read sequence for the alignment (to avoid the adapter sequence that may be present in the read sequence). We allowed up to two mismatches and up to three alignments of same quality per read sequence. Reads that aligned at more than three unique genomic locations were removed from further analysis to avoid repetitive elements. Fold changes were calculated as a ratio of total number of reads in the control divided by the total number of reads in

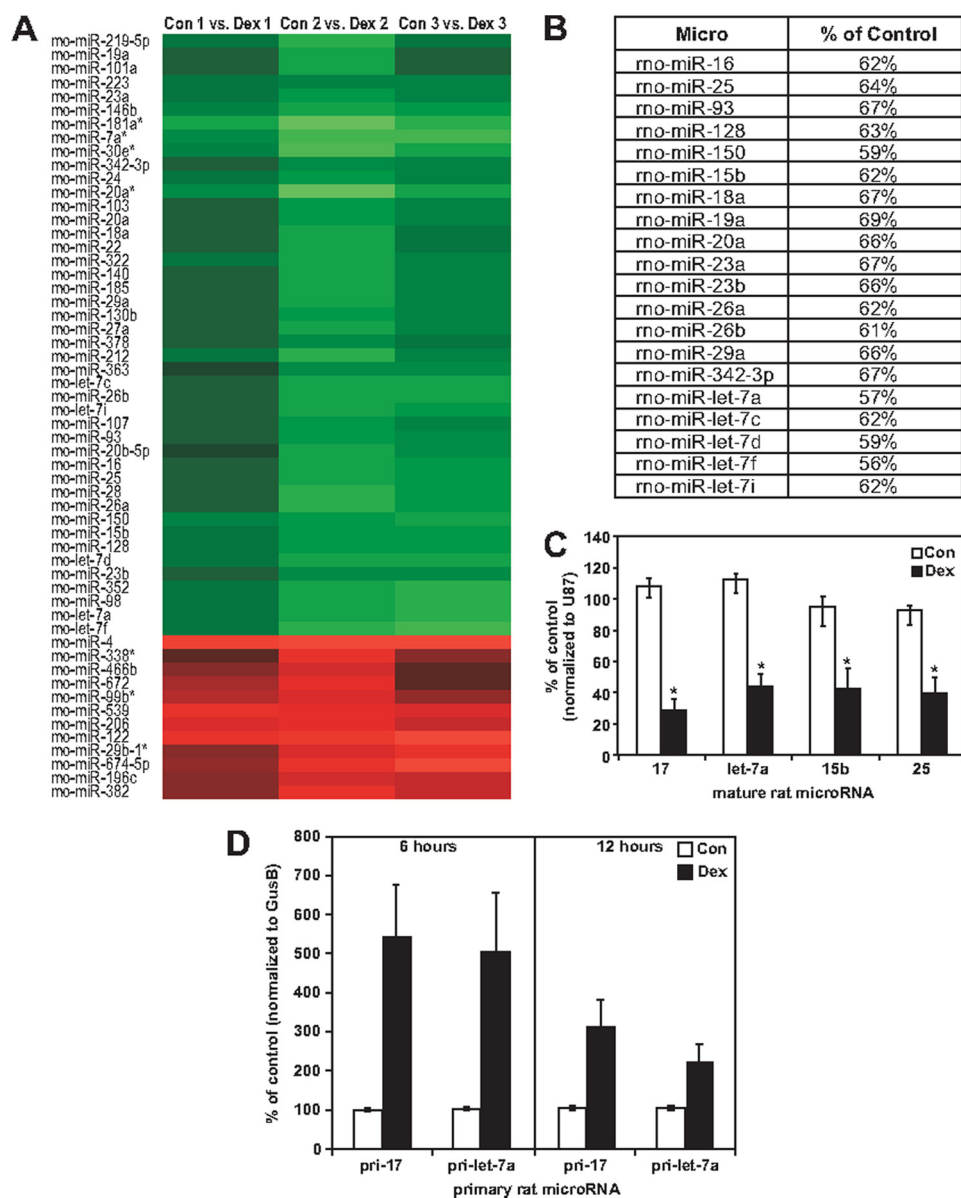


FIGURE 2. Repression of microRNA expression during glucocorticoid-induced apoptosis of primary thymocytes. Primary thymocytes were isolated and treated with 100 nM dexamethasone for 6 h. Total RNAs were subjected to microRNA microarray analysis on the Agilent rat genome microRNA array. *A*, heat map representation of microRNAs differentially expressed in control (Con) and dexamethasone (Dex)-treated primary thymocytes. *Red* indicates microRNAs induced by dexamethasone treatment, and *green* indicates microRNAs repressed (three independent biological replicates, $p < 0.05$). *B*, percentage of repression of abundantly expressed microRNAs (Micro, basal intensity values > 200). The results are calculated as intensity dexamethasone-treated/intensity untreated. *C*, quantitative PCR validation of microRNA microarray. MicroRNA microarray results were validated by quantitative PCR analysis of three microRNAs significantly regulated on the array as well as one microRNA (rno-miR-17) not significantly regulated on the array. The results are expressed as percentages of control mean values \pm S.E. for three independent experiments. *, $p \geq 0.05$. *D*, quantitative PCR analysis of primary microRNA expression. rno-miR-17 and rno-miR-let-7a primary microRNA transcript expression was analyzed via quantitative PCR and normalized to the GusB housekeeping gene. The results are expressed as percentages of control mean values \pm S.E. for three independent experiments.

dexamethasone treatment, as implemented in Erange (17) for sites that contained at least 20 aligned reads in either of the two samples. Sites that were within 100 bp of a previously annotated microRNA and displayed fold change of 2.0 or greater in either direction were selected for detailed analyses.

Quantitative PCR Analysis—Total RNAs were isolated using the Ambion mirVana miRNA isolation kit (Austin, TX). For mature microRNA and primary microRNA analysis, total

RNAs were reverse transcribed using the TaqMan microRNA reverse transcription kit and analyzed using TaqMan mature microRNA and primary microRNA assays (Applied Biosystems). For gene expression analysis, total RNAs were reverse transcribed using the TaqMan reverse transcription kit and analyzed with TaqMan Gene Expression Assays (Applied Biosystems). All of the quantitative PCRs were performed on a 7900HT quantitative PCR platform (Applied Biosystems).

Western Blotting and Antibodies—The cells were harvested and lysed in 1 \times Laemmli buffer containing 2.5% β -mercaptoethanol (Bio-Rad). Whole cell lysates (20–30 μ g) were fractionated on 6% or 8% Tris-glycine gels (Invitrogen) and transferred to nitrocellulose membranes. The membranes were probed overnight with the following antibodies: Dicer rabbit polyclonal, 1:200 (Santa Cruz Biotechnology, Santa Cruz, CA); Drosha rabbit polyclonal, 1:200 (Santa Cruz Biotechnology); DGCR8/Pasha goat polyclonal, 1:1000 (Abnova, Taipei City, Taiwan); and β -actin, 1:10,000 (Millipore, Billerica, MA).

Apoptosis Assays—Apoptosis was evaluated via annexin V/propidium iodide staining or propidium iodide staining alone. For annexin V analysis, the cells were stained with annexin V according to the manufacturer's protocol and counterstained with propidium iodide (Trevigen, Gaithersburg, MD). Caspase-3 activation was monitored via CaspaTag fluorescence, and the cells were stained according to manufacturer's instructions (Millipore). For propidium iodide staining alone, propidium iodide (Invitrogen) was added to achieve a final concentration of 10 μ g/ml.

Following staining, the cells were immediately analyzed on a Becton Dickinson FACSsort cytometer equipped with CellQuest software.

Animals—Adrenalectomized hsa-miR-17–92 transgenic mice or C57BL/6 wild-type controls were obtained from Jackson Labs (Bar Harbor, ME). Adrenalectomized rats were obtained from Charles River Laboratories (Wilmington, MA). All of the experiments were performed in accordance with the

A	Treatment	Dex	Control
	Total number of reads	13,087,842	12,307,015
	Number of unique reads	2,115,004	941,336
	Mismatches allowed	2	2
	Multi alignments allowed	3	3
	# of Unique reads aligned	1,163,014	543,026
	# of Total reads aligned	10,882,548	9,682,142
	% of reads aligned	83.1%	78.6%

B	Direction of regulation	Number of enriched sites	Enriched sites within 100bp of known microRNA
	MicroRNAs repressed (Con vs. Dex)	1784	132
	MicroRNAs induced (Dex vs. Con)	238	0

C	MicroRNA	% of Control
	rno-miR-16	33%
	rno-miR-25	21%
	rno-miR-93	35%
	rno-miR-128	ND
	rno-miR-150	26%
	rno-miR-15b	34%
	rno-miR-18a	26%
	rno-miR-19a	27%
	rno-miR-20a	24%
	rno-miR-17	19%
	rno-miR-19b	25%
	rno-miR-92a	26%
	rno-miR-23a	22%
	rno-miR-23b	16%
	rno-miR-26a	38%
	rno-miR-26b	34%
	rno-miR-29a	18%
	rno-miR-342	17%
	rno-miR-let-7a	31%
	rno-miR-let-7c	48%
	rno-miR-let-7d	44%
	rno-miR-let-7f	49%
	rno-miR-let-7i	ND

FIGURE 3. MicroRNA deep sequencing confirms the prevalent repression of microRNA expression during glucocorticoid-induced apoptosis. Primary thymocytes were isolated and treated with 100 nM dexamethasone (Dex) for 6 h. Total RNAs were subjected to microRNA deep sequencing analysis. **A**, deep sequencing of two biological replicates was performed using the Illumina Solexa platform for each treatment group, and the resulting sequences from replicates were combined for further analyses. Sequences were aligned to the reference rat genome and sites that were enriched with reads were selected for further analysis. **B**, peak detection and ratio calculation revealed prevalent down-regulation of microRNAs in response to dexamethasone treatment. The ratios were calculated as control *versus* dexamethasone (microRNAs repressed) and dexamethasone *versus* control (microRNAs induced). **C**, percentage of repression of abundantly expressed microRNAs correlates with microRNA microarray results. The results are calculated as intensity dexamethasone-treated/intensity untreated presented as percentages of control. ND denotes microRNAs detected by microarray analysis but not deep sequencing analysis. MicroRNAs highlighted in gray indicate a subset of microRNAs repressed in deep sequencing analysis but not microarray analysis.

NIEHS, National Institutes of Health Institutional Animal Care and Use Committee.

RESULTS

Repression of MicroRNA Expression during Glucocorticoid-induced Apoptosis—For these studies, primary rat thymocytes, a classic model of glucocorticoid-induced apoptosis, were cultured *in vitro* in the presence or absence of the synthetic glucocorticoid dexamethasone for 6 h. Apoptotic progression was monitored in control and glucocorticoid-treated cells via flow cytometric analysis of phosphatidylserine exposure, plasma membrane integrity, and caspase-3 activation. Following 6 h of glucocorticoid treatment, we observed an apoptotic population of cells. These cells exhibited phosphatidylserine externalization as determined by a significant increase in annexin-FITC fluorescence and decreased plasma membrane integrity demonstrated by a modest increase in propidium iodide fluorescence (Fig. 1A). These cells also exhibited an increase in caspase-3 activity determined by an increase in CaspaTag fluorescence, indicating the clear induction of apoptosis following 6 h of glucocorticoid treatment (Fig. 1B). These apoptotic indicators persisted following 12 h of treatment, resulting in significant cell death (Fig. 1). Therefore, we performed our

microRNA expression analysis early in the apoptotic process (prior to cell death) after 6 h of glucocorticoid treatment.

Following 6 h of treatment, total RNAs from control and glucocorticoid-treated cells were prepared and subjected to microRNA microarray analysis. Of the 350 microRNAs represented on our chip, 56 were differentially expressed in response to glucocorticoid-induced apoptosis (Fig. 2A). Surprisingly, the majority of these microRNAs (79% or 44 microRNAs) were repressed, and only 12 low abundance microRNAs were weakly induced during this time frame. Given the low basal expression levels of these induced microRNAs, we were unable to confirm their up-regulation via quantitative PCR. Because microRNA expression levels can vary widely, we focused our efforts on the abundantly expressed and thus potentially biologically active microRNAs (basal intensity values >200). The percent repression values (intensity glucocorticoid/intensity control) were generated for these microRNAs (Fig. 2B). Interestingly, these abundantly expressed microRNAs were repressed uniformly irrespective of their genomic

locations, suggesting that the repression of microRNA expression during glucocorticoid-induced apoptosis occurs post-transcriptionally, perhaps at the level of bioprocessing. The repression of a subset of microRNAs identified as differentially expressed (rno-let-7a, rno-miR-15b, and rno-miR-25) as well as the repression of a microRNA not significantly regulated in the microarray (rno-miR-17) was validated via quantitative PCR analysis (Fig. 2C). Interestingly, the expression of rno-miR-17 and rno-let-7a primary transcripts was increased during glucocorticoid-induced apoptosis, providing important evidence that the repression of the resultant mature microRNAs occurs post-transcriptionally (Fig. 2D).

To evaluate the expression of the microRNAome during glucocorticoid-induced apoptosis by an alternative approach, we performed next generation sequencing or deep sequencing of microRNAs from the control and glucocorticoid-treated samples. These samples were obtained from independent animals rather than those described in Fig. 2; however, these cells exhibited apoptotic kinetics similar to those described in Fig. 1. Deep sequencing of microRNAs is an open-ended and hybridization-independent approach that facilitates the detection of both annotated and novel microRNAs. In our study, massively parallel sequencing of control and glucocorticoid-treated RNA

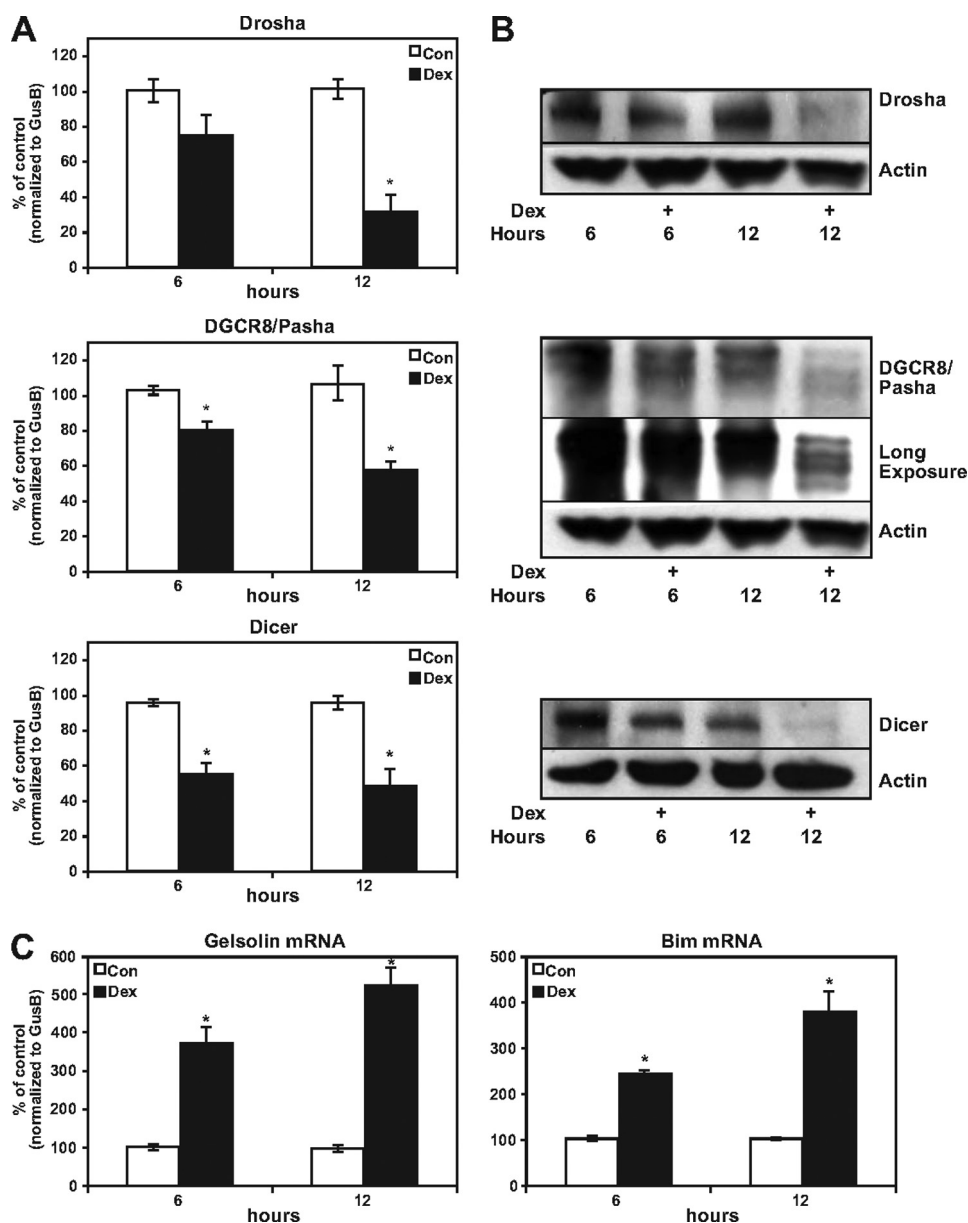


FIGURE 4. MicroRNA processing enzymes are repressed during glucocorticoid-induced apoptosis of primary thymocytes. Primary thymocytes were isolated and treated with 100 nM dexamethasone (Dex) for 6 and 12 h. *A*, the mRNA expression of the microRNA processing enzymes Drosha, DGCR8/Pasha, and Dicer was evaluated by quantitative PCR and normalized to the GusB housekeeping gene. The results are expressed as the percentages of control (Con) mean values \pm S.E. for three independent experiments. $^* p \geq 0.05$. *B*, whole cell lysates were immunoblotted with Drosha, DGCR8/Pasha, or Dicer polyclonal antibodies. Actin immunoblotting served as a loading control, and the results are representative of at least three independent experiments. *C*, induction of Bim and Gelsolin mRNA expression during glucocorticoid-induced apoptosis. Primary thymocytes were isolated and treated with 100 nM dexamethasone for 6 and 12 h. The mRNA expression of Bim and Gelsolin was evaluated by quantitative PCR and normalized to the GusB housekeeping gene. The results are expressed as the percentages of control mean values \pm S.E. for three independent experiments. $^* p \geq 0.05$.

samples generated millions of short sequences (commonly referred to as “reads”), which were aligned to the reference rat genome (Fig. 3A). We identified a set of 1782 sites (genomic loci that may be encoding microRNAs) that were down-regulated and 238 sites that were up-regulated by glucocorticoid treatment (see “Experimental Procedures” for more details). Of the 1782 sites, 132 sites were within 100 bp of an annotated microRNA, which correspond to 129 individual microRNAs (supplemental Table S1). It is also possible that the sites not immediately adjacent to a known microRNA may house novel

microRNAs, which warrants further evaluation. Similar to the aforementioned microarray study, we detected a number of sites that were up-regulated by dexamethasone treatment; however, none of the 238 up-regulated sites were in close proximity to an annotated microRNA (Fig. 3B). These results obtained by deep sequencing mirror the microarray findings of prevalent microRNA repression and minimal microRNA induction during glucocorticoid-induced apoptosis of primary thymocytes. In addition, the percent repression values of abundantly expressed microRNAs generated using deep sequencing data were comparable with those generated using microarray data, further suggesting the post-transcriptional repression of microRNAs during glucocorticoid-induced apoptosis of lymphocytes (Fig. 3C). Deep sequencing analysis confirmed the repression of 35 of the 44 microRNAs repressed in the microarray analysis (79%). Moreover, microRNA deep sequencing was also able to detect repressed microRNAs not detected by microarray (representative values in gray in Fig. 3C), suggesting that deep sequencing of microRNAs is a more sensitive technique that may be used in conjunction with microRNA microarray and quantitative PCR as a novel “weight of evidence” approach to comprehensive profiling of the microRNAome.

MicroRNA Processing Enzymes Are Dysregulated during Glucocorticoid-induced Apoptosis—The broad, genome-wide down-regulation of microRNA expression detected by both microarray and deep sequencing analysis and the absence of primary microRNA trans-

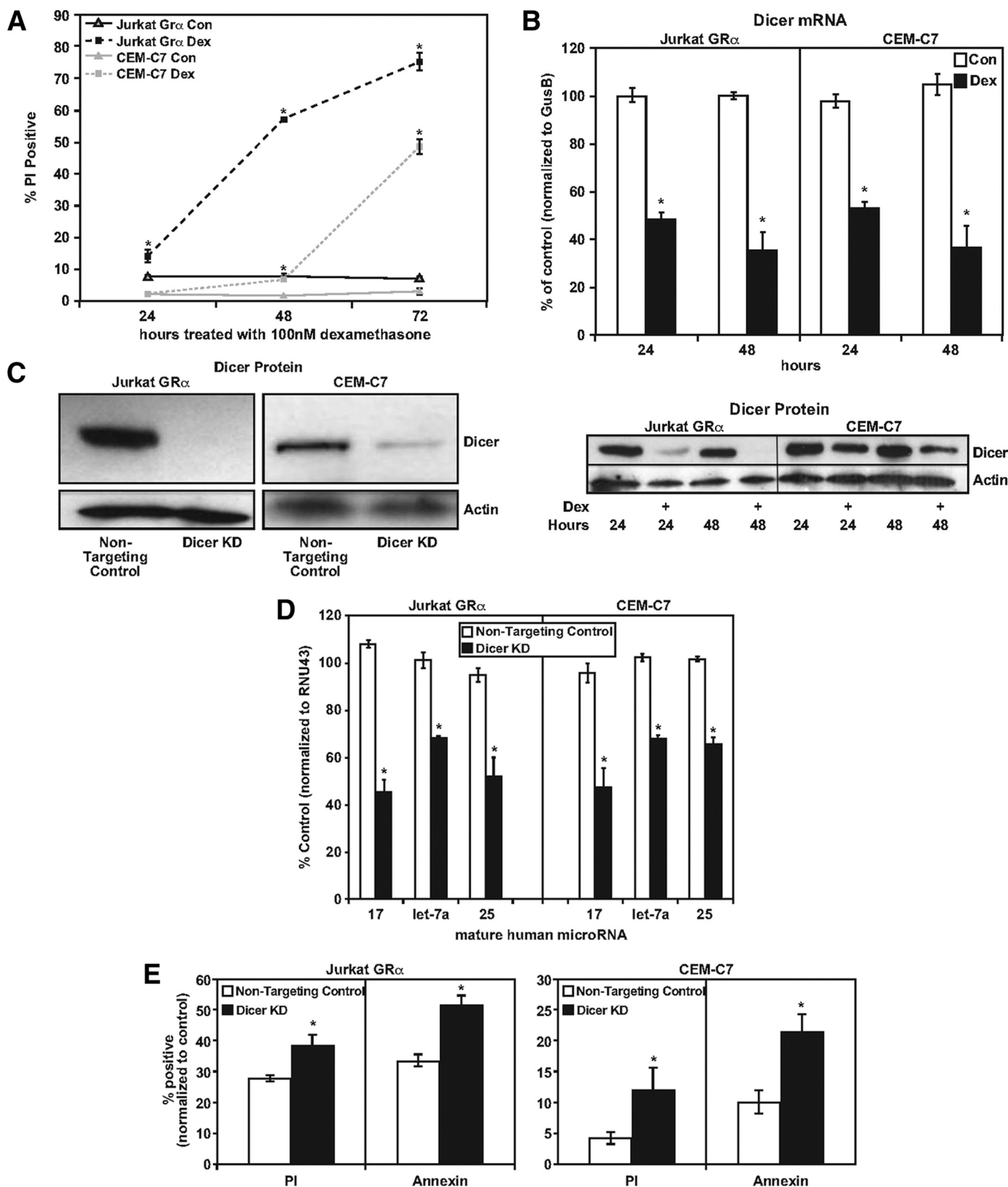
cript repression suggest post-transcriptional repression of mature microRNAs during glucocorticoid-induced apoptosis of lymphocytes. To evaluate this hypothesis, we examined the expression of the key microRNA processing enzymes Drosha, DGCR8/Pasha, and Dicer during glucocorticoid-induced apoptosis of primary thymocytes. Following 6 h of glucocorticoid treatment, the mRNA expression of each processor is repressed, with the repression of Dicer and DGCR8/Pasha mRNA achieving statistical significance (Fig. 4A). After 12 h of treatment, the mRNA levels of each processor are significantly

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repressed in response to glucocorticoid-induced apoptosis (Fig. 4A). The reduced expression of microRNA processing enzymes is not due to an apoptosis-related global increase in RNase activity because the expression of the glucocorticoid-respon-

sive genes *Gelsolin* and *Bim* are strongly induced under these conditions in our cells (Fig. 4C).

The repression of microRNA processing enzymes was also evident at the level of protein expression (Fig. 4B). *Drosha* pro-



tein expression was decreased following 6 h of glucocorticoid treatment and was essentially undetectable at 12 h. The expression of Droscha's co-factor DGCR8/Pasha was also reduced in response to glucocorticoid treatment and upon overexposure, a clear cleavage pattern emerged following 12 h of treatment. Furthermore, the protein expression of the cytoplasmic processor, Dicer, was also markedly reduced following 6 h of glucocorticoid treatment, whereas at 12 h full-length Dicer was barely detectable (Fig. 4C). This repression of the microRNA bioprocessing pathway results in the nuclear accumulation of unprocessed primary microRNAs (supplemental Fig. S1), accounting for the increased expression of microRNA primary transcripts observed during glucocorticoid-induced apoptosis (Fig. 2D).

MicroRNA Repression Enhances Glucocorticoid-induced Apoptosis of Lymphocytes—To assess whether the repression of microRNA expression contributes to the cell death process, we silenced the expression of Dicer, the enzyme responsible for mature microRNA biosynthesis, in two human lymphoid cell lines. The transition to cultured cell lines was necessary because primary rat thymocytes are difficult to genetically manipulate *in vitro*. Therefore, we performed our functional analyses in the Jurkat GR α and the CEM-C7 leukemic cell lines. The Jurkat GR α cell line was generated by the exogenous expression of the full-length GR α isoform in the glucocorticoid-resistant Jurkat ALL parental cell line (18), and stable expression of GR α restores glucocorticoid sensitivity to Jurkat cells (Fig. 5A). The glucocorticoid-sensitive CEM-C7 cell line contains an endogenous, hormone-responsive GR α and exhibits slower apoptotic kinetics in response to glucocorticoid compared with the Jurkat GR α cell line (Fig. 5A). Interestingly, similar to the results obtained in primary cell culture, Dicer expression is repressed at both the mRNA and protein levels in each leukemic cell line during glucocorticoid-induced apoptosis (Fig. 5B). Stable Dicer depletion in these cells was achieved via lentiviral transduction of shRNA (Fig. 5C), and the depletion of Dicer resulted in the reduced expression of a subset of microRNAs (Fig. 5D). Dicer depletion and the subsequent repression of microRNA expression augmented glucocorticoid induced apoptosis in both cell lines as determined by flow cytometric evaluation of phosphatidylserine exposure and plasma membrane integrity (Fig. 5E). Importantly, this increased sensitivity to glucocorticoid-induced apoptosis was not due to increased GR expression in the absence of Dicer expression (data not shown). These data sug-

gest that Dicer depletion and subsequent microRNA repression enhance the progression of glucocorticoid-induced apoptosis in lymphocytes.

Overexpression of the Repressed miR-17-92 Polycistron Blunts Glucocorticoid-induced Apoptosis—To delineate the potential role of specific microRNAs down-regulated during glucocorticoid-induced apoptosis, we sought to overexpress the anti-apoptotic miR-17-92 polycistron. Members of this polycistron were repressed by glucocorticoid treatment in primary thymocytes, as determined by both the microarray and deep sequencing studies. Furthermore, mature members of this polycistron are also down-regulated during glucocorticoid-induced apoptosis of cultured leukemic cells (Fig. 6A). Overexpression of the hsa-miR-17-92 polycistron via lentiviral transduction of hsa-miR-17-92 precursor microRNA resulted in efficient overexpression of individual mature hsa-miR-17-92 polycistron members in the Jurkat GR α cell line (Fig. 6B). Overexpression of hsa-miR-17-92 polycistron members in the Jurkat GR α background significantly diminished the ability of both subsaturating and saturating doses of glucocorticoid to induce glucocorticoid-induced apoptosis (Fig. 6C). This diminished sensitivity was not due to decreased GR expression in response to hsa-miR-17-92 overexpression (data not shown). In addition, primary thymocytes derived from mice transgenic for the hsa-miR-17-92 polycistron (~1.5–2-fold overexpression of mature hsa-miR-17-92 polycistron members (8)) in the lymphocyte compartment exhibited decreased sensitivity to glucocorticoid-induced apoptosis (Fig. 6D). This decreased sensitivity to glucocorticoid-induced apoptosis may also contribute to the hematolymphoid phenotype of these mice. These data suggest that the specific repression of miR-17-92 polycistron contributes to glucocorticoid-induced apoptosis of lymphocytes *in vitro* and *in vivo*.

DISCUSSION

Glucocorticoid-induced apoptosis is a key component of thymocyte development. Furthermore, because of their potent apoptosis-inducing properties, synthetic glucocorticoids are common components of hematolymphoid chemotherapy regimens. Here we provide evidence of broad microRNA repression during glucocorticoid-induced apoptosis of lymphocytes. This repression was associated with the reduction of both nuclear (Droscha and DGCR8/Pasha) and cytoplasmic (Dicer)

FIGURE 5. MicroRNA depletion contributes to glucocorticoid-induced apoptosis in human leukemic cell lines. A, flow cytometric analysis of glucocorticoid-induced apoptosis in Jurkat GR α and CEM-C7 ALL leukemic cell lines. The cells were treated with 100 nM dexamethasone (Dex), and apoptotic progression was monitored at 24, 48, and 72 h via flow cytometric analysis of propidium iodide (PI) uptake. The results are represented as the mean percentages of PI-positive values \pm S.E. for six independent experiments. *, $p \geq 0.05$. B, repression of Dicer expression during glucocorticoid-induced apoptosis of human leukemic cell lines. The cells were treated with 100 nM dexamethasone for 24 and 48 h. The expression of Dicer mRNA was evaluated in total RNA via quantitative PCR. The expression of GusB mRNA served as an endogenous control (Con). The results are represented as percentages of control mean values \pm S.E. for three independent experiments. *, $p \geq 0.05$. Whole cell lysates were immunoblotted with Dicer polyclonal antibody. Actin immunoblotting served as a loading control. The results are representative of three independent experiments. C, stable silencing of Dicer protein expression in Jurkat GR α and CEM-C7 lymphocyte cell lines via lentiviral transduction of shRNA. Whole cell lysates from cells stably transduced with nontargeting control or anti-Dicer shRNA (Dicer knock down (KD)), were immunoblotted with Dicer polyclonal antibody. Actin immunoblotting served as a loading control, and the results are representative of three independent experiments. D, repression of mature microRNA expression in the absence of Dicer expression. Total RNAs from nontargeting control and Dicer KD cells were evaluated for the expression of three independent mature microRNAs via quantitative PCR. The expression of Rnu43 small nuclear RNA served as an endogenous control. The results are reported as percentages of nontargeting control values \pm S.E. for three independent experiments. *, $p \geq 0.05$. E, enhancement of glucocorticoid-induced apoptosis in Dicer KD cells. Flow cytometric analysis of annexin-FITC and propidium iodide uptake in nontargeting control and Dicer KD stable cell lines in response to 48 h of 100 nM dexamethasone treatment. The results were normalized to untreated control and are representative of three independent experiments. *, $p \geq 0.05$.

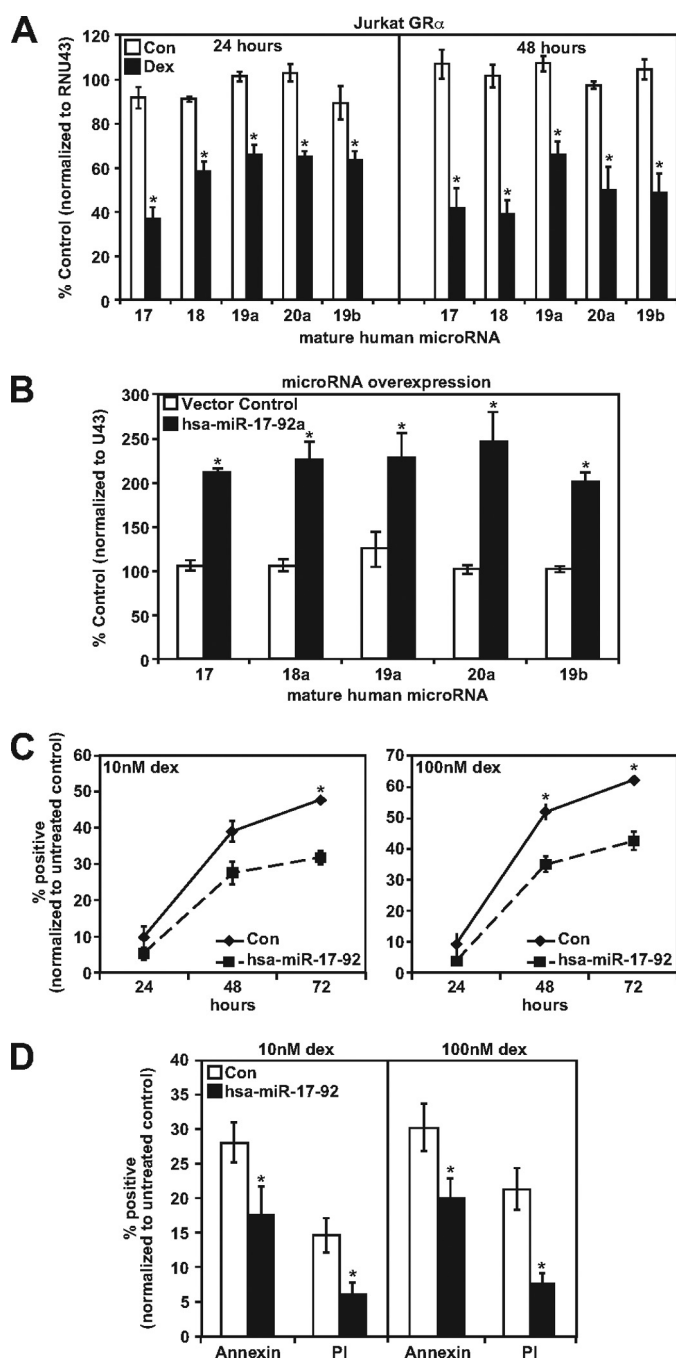


FIGURE 6. Overexpression of hsa-miR-17-92 blunts glucocorticoid-induced apoptosis. A, repression of hsa-miR-17-92 expression during glucocorticoid-induced apoptosis of Jurkat GR α cells. Jurkat GR α cells were treated with 100 nM dexamethasone (Dex) for 24 and 48 h. The expression of individual mature hsa-miR-17-92 polycistron members was evaluated via quantitative PCR. The expression of Rnu43 small nuclear RNA served as an endogenous control (Con). The results are represented as mean percentages of control values \pm S.E. for three independent experiments. *, $p \geq 0.05$. B, efficient overexpression of individual polycistron members in Jurkat GR α cells via lentiviral transduction. Jurkat GR α cells were stably transduced with lentivirus encoding the hsa-miR-17-92 precursor microRNA or empty vector control. The overexpression of individual mature hsa-miR-17-92 polycistron members was evaluated via quantitative PCR. The expression of Rnu43 small nuclear RNA served as an endogenous control. The results are represented as mean percentages of vector control values \pm S.E. for three independent experiments. *, $p \geq 0.05$. C, Hsa-miR-17-92 overexpression blunts glucocorticoid-induced apoptosis. Flow cytometric analysis of propidium iodide (PI) uptake in empty vector or hsa-miR-17-92 stable overexpressors in response to 10 nM and 100 nM dexamethasone treatment. The results were normalized to untreated control and are representative of three independent

microRNA processing enzymes and the increased expression of primary microRNA transcripts.

Dicer repression and cleavage during apoptosis has recently been reported in laboratory-derived human endothelial cells, HL-60 leukemic cells, and HeLa cervical carcinoma cells in response to apoptotic stimuli; however, none of these reports evaluated the expression of the nuclear processors Drosha and DGCR8/Pasha during apoptosis (19–22). This reduction of Dicer protein expression has recently been attributed to caspase-3 cleavage (20). The precise mechanism of Dicer mRNA repression during apoptosis remains undetermined. Interestingly, we show that Dicer repression in response to glucocorticoid-induced apoptosis is conserved in both healthy and transformed lymphocytes. Therefore, the results of this study expand our current understanding of Dicer repression in response to apoptotic stimulation while being the first to detail the repression of all three key microRNA processing enzymes during glucocorticoid-induced apoptosis of lymphocytes. Additional studies in our laboratory have revealed that alternative apoptotic stimuli, specifically Fas ligand and UVC, also promote the repression of microRNA expression and processing enzymes during primary thymocyte apoptosis, suggesting that the dysregulation of microRNA expression and bioprocessing machinery is a common convergence point for multiple apoptotic pathways during apoptosis of lymphocytes.³

Several recent reports have suggested that sex steroid hormones also exert post-transcriptional control through the regulation of microRNA processing and expression. For example, ligand-bound estrogen receptor α regulates microRNA expression in cultured MCF-7 breast cancer cells, myometrial, and leiomyoma smooth muscle cells, rat mammary tissue, and mouse splenic lymphocytes (23–28). MicroRNAs can also negatively regulate the estrogen receptor α transcriptional response via translational inhibition of estrogen-responsive genes and the p160 transcriptional co-activator, AIB1 (24, 25). Additionally, estrogen treatment of ovariectomized mice led to the repression of a subset of microRNAs (29). This study found that ligand-activated estrogen receptor α inhibits Drosha processing of precursor microRNAs by mediating the dissociation of the Drosha microprocessor machinery from the precursor microRNA. These results provide additional mechanisms for hormonal impairment of microRNA processing. Alternatively, androgen treatment of androgen-responsive prostate cancer cell lines induced the expression of 16 mature microRNAs in the absence of microRNA repression (30). Further analysis identified direct transcriptional activation of miR-21 via androgen receptor binding to the miR-21 promoter region, indicating that nuclear hormone receptors can also act via classical ligand-

³ L. K. Smith and J. A. Cidlowski, unpublished observations.

experiments. *, $p \geq 0.05$. D, transgenic hsa-miR-17-92 overexpression hinders glucocorticoid-induced apoptosis of primary thymocytes. Primary thymocytes were derived from wild-type control and hsa-miR-17-92 transgenic mice and treated with 10 and 100 nM dexamethasone for 12 h. Apoptosis was monitored by flow cytometric analysis of annexin-FITC/propidium iodide staining. The results were normalized to untreated control and are representative of four independent experiments. *, $p \geq 0.05$.

activated transcription factor signaling to induce microRNA expression.

Previously, a single study sought to examine the expression of mature microRNAs during glucocorticoid-induced apoptosis of lymphocytes. In conflict with our results, this group did not detect the prevalent repression of microRNAs in response to dexamethasone treatment (31). Moreover, this study could not validate the down-regulation of any mature microRNA repressed on their array via quantitative PCR and in contrast reported the induction of three microRNAs: hsa-miR-15b, -16, and -223. Interestingly, in our model system we observe the strong repression of rno-miR-15b in response to glucocorticoid-induced apoptosis (Fig. 2C). These discrepancies are likely due to the use of alternative experimental models, different methods of data analysis, and distinct microarray platforms. The findings of a separate analysis indicate that the hormonal regulation of microRNAs is likely specific to cell type and physiological context. For example, glucocorticoid treatment of lung epithelial cells, which do not undergo glucocorticoid-induced apoptosis, failed to significantly alter microRNA expression (32). These data suggest that the dramatic down-regulation of microRNA expression reported in our study is likely due to the physiological activation of the apoptotic cascade in response to glucocorticoid treatment.

We also report that the depletion of Dicer and Dicer-dependent microRNA expression enhanced glucocorticoid-induced apoptosis in two human leukemic cell lines. Dicer has an essential role in the development of the adaptive immune system. Conditional deletion of Dicer expression in the T-cell compartment results in impaired T-cell development and diminished regulatory T-cell function (33–35), and ablation of Dicer expression in the B-cell compartment attenuates B-cell development and alters the antibody repertoire (36). Therefore, Dicer depletion hinders lymphocyte development and increases sensitivity to glucocorticoid-induced apoptosis.

In our studies, the stable (~2 weeks) depletion of Dicer in human leukemic cell lines resulted in the partial (40–60%) reduction of evaluated microRNAs. This partial reduction may be due to the robust stability of mature microRNAs or to the activity of an alternative microRNA processing pathway. In fact, a recent report describes a novel Dicer-independent, Argonaute-2-dependent microRNA processing pathway (37). Interestingly, unlike other microRNA bioprocessors, Argonaute-2 expression is not reduced during glucocorticoid-induced apoptosis of primary thymocytes (supplemental Fig. S2), suggesting that microRNAs refractory to Dicer depletion may be processed in an Argonaute-2-dependent manner during glucocorticoid-induced apoptosis.

Furthermore, we determined that the overexpression of specific microRNAs repressed during glucocorticoid-induced apoptosis of lymphocytes, the miR-17–92 polycistron, blunts glucocorticoid-induced apoptosis in both human and murine-derived lymphocytes. Members of this highly conserved polycistron exhibit complete sequence identity in the human, rat, and mouse, suggesting that their anti-apoptotic functions are conserved across vertebrate species (38). Interestingly, transgenic overexpression of the hsa-miR-17–92 polycistron suppresses expression of the pro-apoptotic Bcl-2 family member,

Bim (8). The induction of Bim expression is a critical component of glucocorticoid-induced apoptosis signaling (39–42). Therefore, decreased basal Bim expression or weakened Bim induction upon glucocorticoid treatment may account for the decreased sensitivity of hsa-miR-17–92-overexpressing lymphocytes to glucocorticoid-induced apoptosis. In summary, these studies establish a “glucocorticoid-induced apoptotic signature” of prevalent repression of microRNAs and microRNA bioprocessing machinery and further delineate the functional significance of this repression.

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